Increases in Mouse Uterine Heat Shock Protein Levels Are a Sensitive and Specific Response to Uterotrophic Agents

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There is increasing consensus that the uterotrophic estrogenicity assay should be coupled with other morphometric or molecular end points that might enhance its sensitivity. We have previously shown that bisphenol A (BPA), similarly to 17β-estradiol (E_2), increases levels of uterine heat shock proteins (hsps), mainly hsp90α and glucose-regulated protein (grp) 94. In this study we investigated whether increases in uterine hsp levels are a specific response of estrogens or estrogen mimics. We therefore examined the ability of *a*) E_2, diethylstilbestrol (DES), and tamoxifen (TAM); *b*) the xenoestrogens coumestrol (CM), methoxychlor (MXC), BPA, and dibutyl phthalate (DBP); *c*) the progestin medroxyprogesterone (MED); *d*) the glucocorticoid dexamethasone (DEX); and *e*) phytol (PHY), a precursor to a retinoid X and peroxisome proliferator-activating receptor agonist, to increase uterine weights and alter uterine morphology and hsp levels. We showed that DES, TAM, CM, MXC, and BPA significantly increased uterine weights and uterine hsp90α and grp94 levels. Even though the doses of CM, MXC, and BPA used were much higher than the E_2 dose, those treatments resulted in lower increases in uterine weight. On the other hand, increases in grp94 levels were equal to those induced by E_2 treatment. Treatments with MED, DEX, DBP, or PHY did not significantly alter uterine weight or morphology and had no significant effects on uterine hsp levels. The results of this study suggest that only the estrogens increase uterine hsp90α and grp94 levels, and that this hsp effect is a more sensitive uterotrophic response than uterine weight increase. *Key words:* bisphenol A, coumestrol, dexamethasone, endocrine disruptors, estrogen, heat shock proteins, methoxychlor, medroxyprogesterone, tamoxifen, uterotrophism. *Environ Health Perspect* 110:1207–1212 (2002). [Online 7 October 2002] *http://ehpnet1.niehs.nih.gov/docs/2002/110p1207-1212papaconstantinou/abstract.html*

Endocrine disruptors are exogenous agents that cause adverse health effects in an intact organism or its progeny consequent to alterations in endocrine function (Olea et al. 1998). A group of these agents act by mimicking the action of estrogens and are thus termed xenoestrogens or estrogen mimics. Examples of these xenoestrogens are the industrial chemicals bisphenol A (BPA), an agent used in the production of polycarbonates and epoxy resins; dibutyl phthalate (DBP), a plasticizer used in the production of PVC and in food packaging; and methoxychlor (MXC), a pesticide manufactured to replace *o,p′*-DDT. Other xenoestrogens are naturally produced by plants and are termed phytoestrogens. These, among others, include coumestrol (CM).

The estrogenicity of these agents has been demonstrated in numerous *in vitro* studies. BPA, MXC, DBP, and CM compete with 17β-estradiol (E_2) for binding to the estrogen receptor (ER). CM has a higher affinity for ERβ than for ERα (Kuiper et al. 1998; Whitten and Patisaul 2001) and is more potent than BPA or MXC for binding to both receptor isoforms and activating transcription through the ER (Kuiper et al. 1998). BPA and MXC have similar affinities for ERα and ERβ (Kuiper et al. 1998). DBP, although less potent than CM, MXC, or BPA, competes with E_2 for binding to the ER (Jobling et al. 1995) and increases reporter gene activity in yeast transfected with the human ER (Harris et al. 1997). BPA, MXC, and DBP display weak estrogenic activities in the E-screen, an assay that measures the proliferation of MCF-7 breast cancer cells in response to treatments with potential estrogen mimics, with the rank order of potency, BPA > MXC > DBP (Andersen et al. 1999).

In vivo MXC, CM, and BPA mimic E_2 actions in the uterus. Increases in uterine weight have been reported for CM (Hunter et al. 1999; Markaverich et al. 1995; Tinwell et al. 2000), MXC (Eroshenko and Cooke 1990; Laws et al. 2000; Mehmood et al. 2000), and BPA (Ashby and Tinwell 1998; Laws et al. 2000; Papaconstantinou et al. 2000). Increases in uterine epithelial cell height have been shown for CM (Medlock et al. 1995; O'Connor et al. 2000), for MXC (Eroshenko and Cooke 1990; Newbold et al. 2001; Rourke et al. 1991), and for BPA (Markey et al. 2001; Papaconstantinou et al. 2000; Steinmetz et al. 1998). In addition, MXC and BPA stimulate several uterine estrogen-regulated genes. For example, MXC increases levels of uterine peroxidase (Cummings and Metcalf 1994; Mehmood et al. 2000), creatinine kinase (Cummings and Metcalf 1995), lactoferrin, glucose-6-phosphate dehydrogenase (Ghosh et al. 1999), and epidermal growth factor receptor (Metcalf et al. 1996). BPA increases levels of uterine insulin growth factor-I mRNA (Klotz et al. 2000), C3 mRNA (Diel et al. 2000), c-*fos* mRNA (Steinmetz et al. 1998), lactoferrin (Markey et al. 2001), progesterone receptor (PR), and peroxidase (Gould et al. 1998).

The addition of morphometric or cellular end points to the rodent uterotrophic assay to enhance its sensitivity has been suggested (Ashby 2001; Newbold et al. 2001). We have previously shown that BPA resembles E_2 in its ability to induce increases in uterine heat shock protein (hsp) levels, mainly hsp90α and glucose-regulated protein (grp) 94 and, to a lesser extent, hsp72 (Papaconstantinou et al. 2001). We also demonstrated that both E_2 and BPA increased levels of hsp90α and grp94 (Papaconstantinou et al. 2001) at doses lower than those necessary for a significant increase in uterine weight (Papaconstantinou et al. 2000). The use of hsps as biomarkers of environmental pertubation shows promise despite some criticisms (Bierkens 2000). Levels of hsps increase in response to cellular injury and protein damage to protect the cell by assisting in the correct folding and processing of proteins (Welch 1987). Hsps are also involved in the signaling of the ER (Pratt 1998) and associate with this receptor (Chambraud et al. 1990; Landel et al. 1995; Sabbah et al. 1996). Glucose-regulated proteins (grps) are hsps found in the endoplasmic reticulum, and their levels increase as a result of protein damage and in response to glucose starvation and calcium depletion (Lee 2001). The ability of estrogen to alter levels of the uterine hsp90 isoforms hsp90α and hsp90β has been demonstrated previously (Shyamala et al. 1989). Estrogen also regulates mRNA levels of uterine hsp70 (Rivera-Gonzalez et al. 1998; Tang et al. 1995; Wu et al. 1996), hsp90, and grp94 (Shyamala et al. 1989).

In this study we evaluated the specificity of the heat shock response in the uterus and

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We thank P. Goering (CDRHCenter for Devices and Radiological Health, U.S. Food and Drug AdministrationFDA) for careful and critical reading of this manuscript.

Received 14 February 2002; accepted 14 May 2002.

determined whether these proteins can be used as an early indicator of uterotrophism. We compared the effects on murine uterine weight, morphology, and hsp levels in response to exogenous treatments with estrogenic substances, including diethylstilbestrol (DES), a synthetic estrogen; tamoxifen (TAM), a compound that behaves as an agonist for most estrogen responses in the uterus; and the environmental estrogen mimics MXC, CM, BPA, and DBP. Furthermore, because the PR and glucocorticoid receptor are thought to have a role in the estrogen-induced increases in uterine weight, especially through effects on the epithelium (Bigsby and Cunha 1988), we examined whether agonists to these receptors alter uterine hsp levels. The agents chosen include the glucocorticoid dexamethasone (DEX), which antagonizes E_2-induced effects in the uterus (Bigsby 1993; Campbell 1978; Sahlin 1995), and the progestin medroxyprogesterone (MED), which has higher affinity for the PR than the natural ligand progesterone and no affinity for the ER (Terenius 1974). Finally, because the retinoid X receptor (RXR) and the peroxisome proliferator-activated receptor α (PPARα) can activate estrogen-responsive genes (Nunez et al. 1997), phytol (PHY), a proagonist for the RXR (Kitareewan et al. 1996) and the PPARα (Ellinghaus et al. 1999; Lampen et al. 2001), was also included in this study.

Materials And Methods

Chemicals. Corn oil (CO; vehicle control), E_2, TAM, DES, DEX, MXC (95% purity), DBP, BPA, MED, and PHY (60% purity) were purchased from Sigma Chemical Co. (St. Louis, MO). CM was purchased from Indofine Chemical Co. (Belle Mead, NJ).

Animals and treatment protocols. All procedures requiring the use of animals were conducted according to the National Institutes of Health *Using Animals in Intramural Research: Guidelines* (1998) as previously described (Papaconstantinou et al. 2000). Ovariectomized female B6C3F1 mice supplied from Charles River Laboratories Inc. (Wilmington, MA) were randomly distributed into treatment groups and housed in groups of four to six in polypropylene cages with stainless steel wire lids and heat-treated chips (Cellu-Dri; Shepherd Specialty Papers, Kalamazoo, MI), with access to food (Lab Rodent Diet 5001; PMI Nutrition International Inc., St. Louis, MO) and water *ad libitum*. The animal rooms were maintained on a 12-hr light/dark cycle (0600 to 1800 hr) at 23 ± 1°C with 30–50% relative humidity.

After an acclimation period of 1–2 weeks, animals were dosed subcutaneously (sc) once a day and for four consecutive days with solutions of CO (vehicle control), E_2 2 µg/kg/day, TAM 1 mg/kg/day, DES 40 µg/kg/day, DEX 1 mg/kg/day, MXC 100 mg/kg/day, CM 10 mg/kg/day, DBP 5,000 mg/kg/day, BPA 100 mg/kg/day, MED 2 mg/kg/day, or PHY 600 mg/kg/day. Doses for E_2 and BPA were based on our previous observation of increases in uterine weight (Papaconstantinou et al. 2000) and hsp levels (Papaconstantinou et al. 2001). The doses for TAM (Carthew et al. 1999b), DES (Farmakalides and Murphy 1984), DEX (Bigsby and Young 1993), MXC (Laws et al. 2000), and CM (Tinwell et al. 2000) were based on previous studies of uterotrophic effects. The dose of DBP was the highest that we were able to administer and exceeded the highest dose of 2,000 mg/kg reported by Zacharewski et al. (1998), which showed no effect on uterine weight. The dose of MED was based on our preliminary dose–response experiment. Finally, because no report exists on the effects of PHY on the uterus, the dose of this chemical was the highest that could be administered. Mice of the first study, which included treatments with E_2, TAM, DES, and DEX, were 36 days old at the start of the treatment period, whereas mice in the second study, which included treatments with E_2, MXC, CM, DBP, BPA, MED, and PHY, were 49 days old at the start of the experiment. Animals were terminated 24 hr after the last treatment with carbon dioxide anesthesia followed by cervical dislocation.

Uterine weight measurements and uterine histology. Mice were weighed to the nearest 0.1 g on day 1 and on the day of termination. No significant differences were observed in animal weights at the beginning or at the end of the treatment period or in feeding behaviors between treatment groups during the experiment. Upon termination, uteri were removed, blotted, and weighed to the nearest 0.1 mg. A 5-mm posteriormost segment of one uterine horn was fixed overnight in 10% neutral buffered formalin, dehydrated in a graded ethanol series, cleared in xylene, embedded in paraplast, sectioned at 8 µm, and stained with hematoxylin and eosin. Photographs of uterine sections were taken on an Olympus BH microscope equipped with an Olympus C-35A camera (Olympus, Woodbury, NY) containing Kodak Ektachrome 160T 35mm film (Eastman Kodak Company, Rochester NY).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The uterus remaining after a section was removed for histology was pooled with the other 3–5 uteri of the same treatment group to obtain enough tissue sample needed for the protein assay, Coomassie staining, and Western blot analyses. There were two groups per treatment. The pooled uteri were homogenized with 0.5 µL of 10 mM Tris buffer, pH 7.4, per milligram of tissue. The homogenates were centrifuged at 15,000 × *g* for 10 min. The postmitochondrial supernatants (Fowler et al. 1989) were collected, and the total protein concentrations of the supernatants were determined by the method of Lowry et al. (1951) with bovine serum albumen (Sigma) as the standard.

Uterine homogenate supernatants were diluted to 3.5 mg protein/mL, and 3 µL of sample (i.e., 10.5 µg/lane) were separated by 12.5% homogeneous sodium dodecyl sulfate-polyacrylamide gel electrophoresis using the Pharmacia Phast System (Pharmacia Biotechnologies, Piscataway, NJ) as described by Goering et al. (1992). Equal loading was verified by Coomassie staining (Pharmacia) of proteins in representative gels.

Immunologic detection and densitometric analysis of heat shock proteins. Electrophoretic transfer of proteins onto nitrocellulose membranes and immunochemical detections of hsp72, hsp90α, hsp73, and grp94 were performed as described by Goering et al. (1992). Briefly, blots were blocked in Tris-buffered saline (TBS: 20 mM Tris, 500 mM NaCl, pH 7.5) with 4% milk for a minimum of 4 hr.

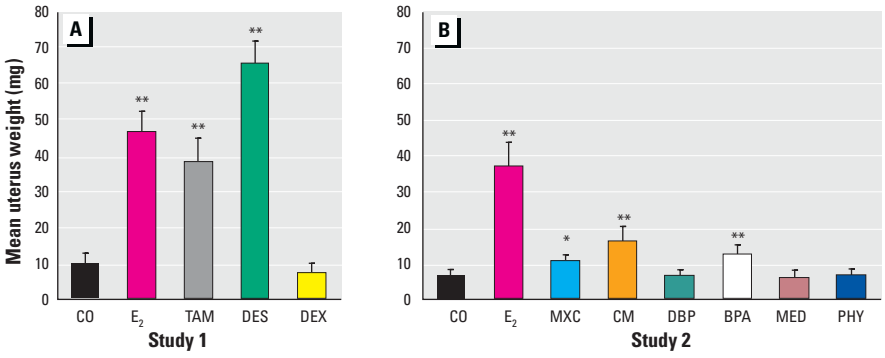

Figure 1. Effects of E_2 (2 µg/kg/day), TAM (1 mg/kg/day), DES (40 µg/kg/day), DEX (1 mg/kg/day), MXC (100 mg/kg/day), CM (10 mg/kg/day), DBP (5,000 mg/kg/day), BPA (100 mg/kg/day), MED (2 mg/kg/day), or PHY (600 mg/kg/day) on uterine weight in ovariectomized B6C3F1 mice. (*A*) Study 1; (*B*) Study 2. Animals were treated sc for four consecutive days. Controls received CO (vehicle control).
*Significant differences from control as determined by one-way ANOVA and the Dunnett's *post hoc* test ($p < 0.05$).
**Significant differences from control as determined by one-way ANOVA and the Dunnett's *post hoc* test ($p < 0.01$).

Solutions of stress protein antibodies (StressGen Biotechnologies, Inc., Vancouver, British Columbia, Canada) at concentrations of 1:500 for hsp72, hsp90α, and grp94, or 1:1,200 for hsp73 were prepared in TTBS (TBS containing 0.05% Tween 20) with 4% milk. Blots were incubated with hsp antibodies overnight followed by a 3-hr incubation with alkaline phosphatase conjugated goat antimouse (Bio-Rad Laboratories, Hercules, CA), goat antirabbit (Sigma), or rabbit antirat (Sigma) secondary antibodies. Blots were scanned with an ARCUS II AGFA scanner, saved as TIFF images, and imported into the Kodak Digital Science 1D Image Analysis Software package (Version 3.0; Eastman Kodak Company) for densitometric analysis. For each homogenate the results of two blots per protein were used to calculate the percent control band intensity. The averages from the two uterine homogenates/treatment were used for the calculation of the means and standard deviations.

Statistical analysis. Results are expressed as means ± standard deviations. One way analysis of variance (ANOVA) was used to assess the variation of the means among the treatments. If the variation was greater than expected by chance alone, Dunnett's test was performed for a comparison of pairs of means. Significance was established when the p-value was less than 0.05.

Results

Uterine weights and histology. Mean uterine weights significantly increased in response to treatments with the estrogens E_2 ($p < 0.01$) and DES ($p < 0.01$), as well as with the mixed estrogen agonist–antagonist TAM ($p < 0.01$, Figure 1). The estrogen-like substances MXC, CM, and BPA significantly increased uterine weights, but to a lesser degree than E_2, DES, or TAM (Figure 1). DEX, a glucocorticoid; MED, a progestin; PHY, a precursor to phytanic acid, an RXR and PPARα activator; and DBP, a weak estrogen and antiandrogen, did not significantly alter uterine weights (Figure 1).

In response to treatment with E_2 or DES, the luminal epithelium changed from simple columnar in control-treated mice (Figure 2A) to pseudostratified, and the thickness of all uterine layers increased (Figure 2B and C, respectively). Treatment with TAM caused hypertrophy of the luminal epithelium and stroma, but did not have a significant effect on the myometrium (Figure 2D). MXC and BPA caused hypertrophy of uterine epithelial cells, and mitoses were present in all uterine layers (Figure 2E and F, respectively). The effects of the phytoestrogen CM on uterine morphology resembled those of E_2 more closely than any of the estrogen-like substances included in the present study. Uterine epithelium height in CM-treated animals increased, with most areas consisting of simple tall columnar epithelium, whereas some had a pseudostratified appearance (Figure 2G). Furthermore, similar to E_2, CM resulted in the presence of secretory granules and pits in the luminal epithelium, vacuoles with degenerating material, and mitotic figures. Uterine morphology in response to treatment with DEX, DBP, MED, and PHY (Figure 2H–K, respectively) was similar to that of CO-treated mice.

Uterine heat shock protein levels. Levels of uterine hsp90α increased in response to all treatments that produced a uterotrophic response (Figure 3A). Of the chemicals examined, E_2, DES, and TAM induced the highest increases in hsp90α. Specifically, the increase of hsp90α levels induced by E_2 in the first study was 15-fold higher than control levels ($p < 0.01$). DES and TAM induced increases to levels approximately 14-fold higher than control ($p < 0.01$). MXC and BPA induced an approximate 5-fold increase, whereas CM induced an approximate 4-fold increase in hsp90α levels compared with control. Levels of uterine hsp90α after treatment with MED were almost double those of controls, but this increase was not statistically significant. Treatment with DEX resulted in a depletion of hsp90α to undetectable levels.

Levels of grp94 were affected by several of the treatments. E_2, TAM, and DES were comparably effective in inducing an increase in grp94 levels (Figure 3B). The E_2-induced increase in grp94 levels compared with control

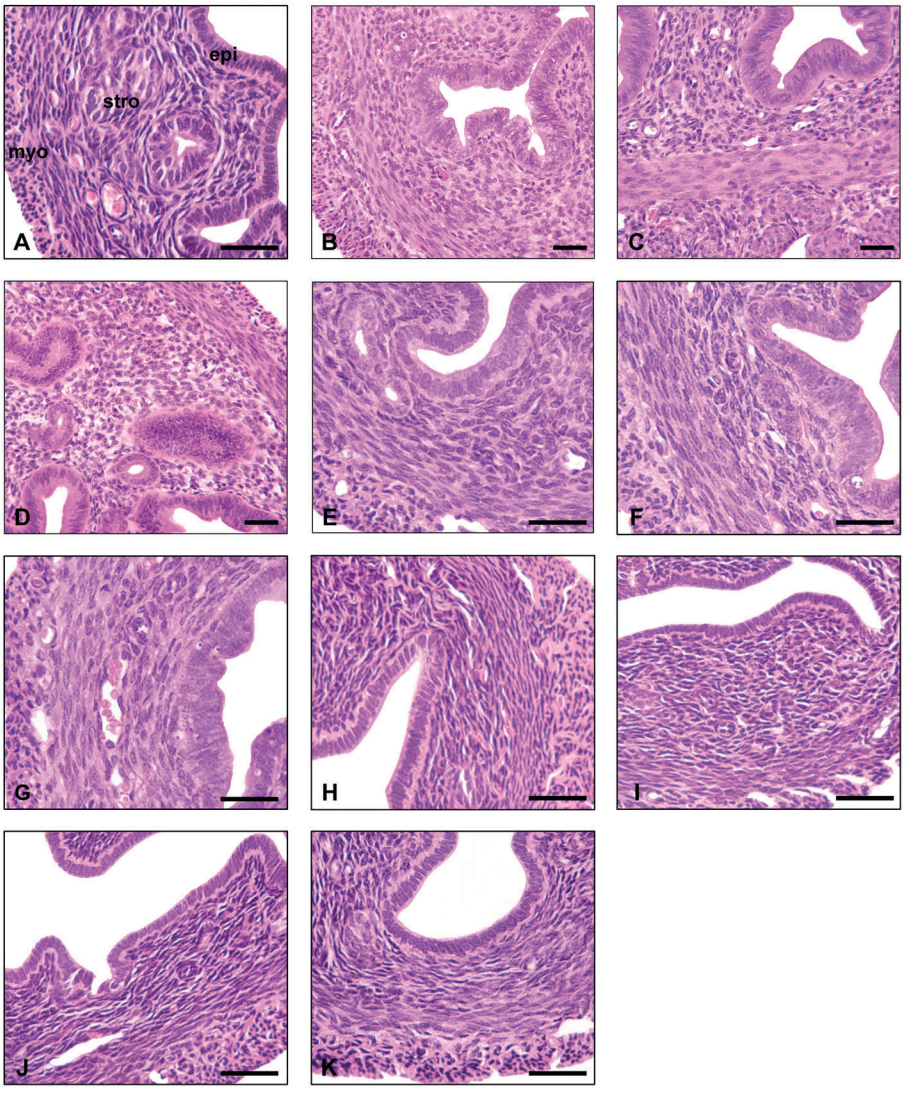

Figure 2. Morphology of the uterus of ovariectomized B6C3F1 mice treated with (*A*) CO; (*B*) E_2 2 µg/kg/day; (*C*) DES 40 µg/kg/day; (*D*) TAM 1 mg/kg/day; (*E*) MXC 100 mg/kg/day; (*F*) BPA 100 mg/kg/day; (*G*) CM 10 mg/kg/day; (*H*) DEX 1 mg/kg/day; (*I*) DBP 5,000 mg/kg/day; (*J*) MED 2 mg/kg/day; or (*K*) PHY 600 mg/kg/day. Epi, stro, and myo in (*A*) designate uterine epithelium, stroma, and myometrium, respectively. Note key histologic changes for E_2 seen in (*B*): increases in uterine epithelial height, stromal, and myometrial thickness. Scale bar = 25 µm.

levels was greater in study 2 than in study 1 ($p < 0.01$ in both studies). MXC, CM, and BPA induced an approximate 4-fold increase in grp94 levels compared with control levels ($p < 0.01$). This increase was equal to that produced by E_2 2 μg/kg/day.

As shown in Figure 4A, E_2 and TAM treatments resulted in 9-fold increases in levels of the hsp70 inducible protein hsp72 compared with control levels ($p < 0.01$). DES induced a slightly lower increase in uterine hsp72 levels. None of the estrogen mimics included in this study effectively increased levels of hsp72. On the other hand, treatments with DEX, DBP, and MED decreased hsp72 levels. Levels of the constitutive hsp70 protein (Figure 4B) hsp73 were slightly increased only by treatment with E_2 in study 2 ($p < 0.05$) and with BPA ($p < 0.05$).

Discussion

In this study we examined the ability of a variety of chemicals to alter uterine morphology and induce hsp levels. These results are summarized in Table 1. Based on the effects listed in this table, TAM 1 mg/kg/day mimicked E_2 2 μg/kg/day on all end points with the exception of the lack of signs of vacuolar degeneration and a nonsignificant increase in hsp73 levels. The ability of TAM to increase mouse uterine weight (Carthew et al. 1999a; Chou et al. 1992) and cause hypertrophy of the uterine epithelium and stroma (Carthew et al. 1999a) has been previously reported. As mentioned earlier, in contrast to E_2, TAM was not an agonist in the myometrium. This is in agreement with a previous study in the rat (Carthew et al. 1999b). An increase in uterine hsp72 mRNA levels in ovariectomized Sprague-Dawley rats treated with TAM 10 mg/kg po has been shown (Rivera-Gonzalez et al. 1998) and supports the increase in hsp72 levels seen in the present study in response to sc treatment with TAM 1 mg/kg/day for 4 days.

As expected from previous studies in B6C3F1 mice (Farmakalides and Murphy 1984), DES mimicked all the uterotrophic responses of E_2. However, no signs of vacuolar degeneration were seen in uterine sections of DES-treated mice. DES induced increases in hsp90α, grp94, and hsp72 levels similar to E_2. MXC, at 100 mg/kg/day, increased uterine wet weight and epithelial height to about one-third that of E_2 2 μg/kg/day. These findings resemble those of Laws et al. (2000) and Mehmood et al. (2000), whose study designs were similar to the one employed in the present study. In addition, MXC significantly increased levels of hsp90α and grp94. CM and BPA exhibited E_2-like effects on the majority of the parameters examined. CM did not induce an increase in hsp73 levels, and neither compound caused significant increases in hsp72 levels. An increase in stromal neutrophils in response to sc treatment with BPA 100 mg/kg/day has been shown previously in immature Alpk-ApfSD rats (Matthews et al. 2001). The increase in grp94 levels induced by MXC 100 mg/kg/day, CM 10 mg/kg/day, or BPA 100 mg/kg/day was the same as that induced by E_2 2 μg/kg/day. The ability of BPA to be as efficacious as E_2 in increasing uterine grp94 levels has been shown previously (Papaconstantinou et al. 2001).

In contrast to the estrogens or estrogen mimics examined, DEX induced nonsignificant decreases in levels of hsp90α and hsp72 and had no effect on uterine weight and morphology. To our knowledge no other study has examined the effect of DEX on hsp levels in the uterus. However, DEX regulates levels of hsp90 and hsp70 in glucose-deprived L929 fibroblast cell cultures (Kasambalides and Lanks 1983). Furthermore, acute DEX treatment of adult rat cardiac myocytes resulted in a small but significant increase in levels of hsp72 (Sun et al. 2000). MED induced a nonsignificant increase in hsp90α levels, decreased hsp72 levels, and had no effect on uterine weight. As in our previous study (Papaconstantinou et al. 2001), the results of this study suggest a correlation between increases in hsp levels and uterine proliferation. This is not surprising, because several studies have shown that hsps can activate protein kinases and other cell-signaling proteins that stimulate cell proliferation and inhibit apoptosis (Kaur et al. 2000; Mizuno et al. 2001; Neckers et al. 1999; Zhang et al. 2001). DEX and progesterone inhibit uterine epithelial cell proliferation in both estrogen-independent (Bigsby and Cunha 1988) and estrogen-dependent (Bigsby 1993; Campell 1978) models. This antiproliferative effect of DEX and progesterone might possibly explain why DEX and

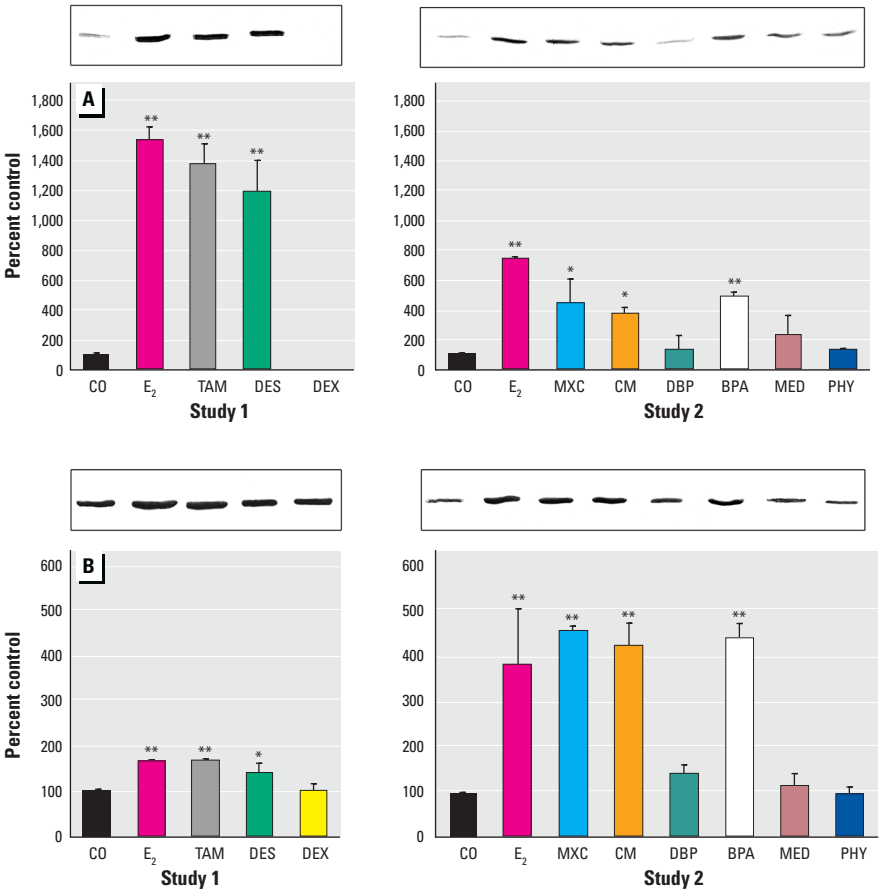

Figure 3. Western analysis of (*A*) hsp90α and (*B*) grp94 protein levels in 15,000 × *g* homogenate supernatants from pooled uterine tissues of ovariectomized B6C3F1 mice. Animals were treated sc for four consecutive days with CO (vehicle control), E_2 (2 μg/kg/day), TAM (1 mg/kg/day), DES (40 μg/kg/day), DEX (1 mg/kg/day), MXC (100 mg/kg/day), CM (10 mg/kg/day), DBP (5,000 mg/kg/day), BPA (100 mg/kg/day), MED (2 mg/kg/day), or PHY (600 mg/kg/day). The bar graphs represent the results of the densitometric analysis of Western blots of hsp90α and grp94. Averages from two Western blots for each protein were calculated. The two averages obtained from the two homogenates per treatment were used for calculation of the means and standard deviations, and the results are expressed as percent of control.
*Statistically significant difference from control as determined by one-way ANOVA and the Dunnett's post hoc test ($p < 0.05$). **Statistically significant difference from control as determined by one-way ANOVA and the Dunnett's post hoc test ($p < 0.01$).

MED treatments in this study decreased levels of hsp72. The subtle nonsignificant increase in hsp90α levels in response to treatment with MED may be an indicator of the potential of this agent to increase uterine weight when given at a dose higher than that used here (Actis et al. 1998). PHY, the precursor to the PPAR and RXR agonist phytanic acid, had no effects on any of the parameters examined, despite several studies that have demonstrated the binding of RXR (Joyeux et al. 1996; Klinge et al. 1997) or the PPAR/RXR heterodimer (Keller et al. 1995) to the estrogen response element and support the ability of these receptors to activate estrogen-responsive genes (Nunez et al. 1997).

Collectively, results of this study demonstrate that all the ER agonists except DBP increased uterine hsp levels. This argues for a role of the ER in the regulation of these genes in the uterus and is supported by our previous observations that E_2 and BPA-induced increases in hsp levels are antagonized by the ER antagonist ICI 182,780 (Papaconstantinou et al. 2001). Whether ER agonists directly affect the transcriptional activity of theses hsp genes is uncertain. Although these genes do not contain estrogen response elements, promoter sequences on many estrogen responsive genes that bind ERs (Beato et al. 1995), they do contain Sp1 promoter sequences (Perry et al. 1994; Vourc'h et al. 1989), which drive ER-mediated transcription of hsp27 (Porter et al. 1997). All chemicals that induced uterine proliferation increased hsp90α and grp94 levels, but not all produced a significant increase in hsp72 levels. Furthermore, MXC, CM, and BPA were as efficacious as E_2 in increasing grp94 levels. This observation, together with the results of our previous study that demonstrated increased levels of grp94 by BPA at doses 40 times lower than those needed to increase uterine weights (Papaconstantinou et al. 2001), suggests that this protein is a more sensitive marker of estrogenic uterotrophic effects than increases in organ weight.

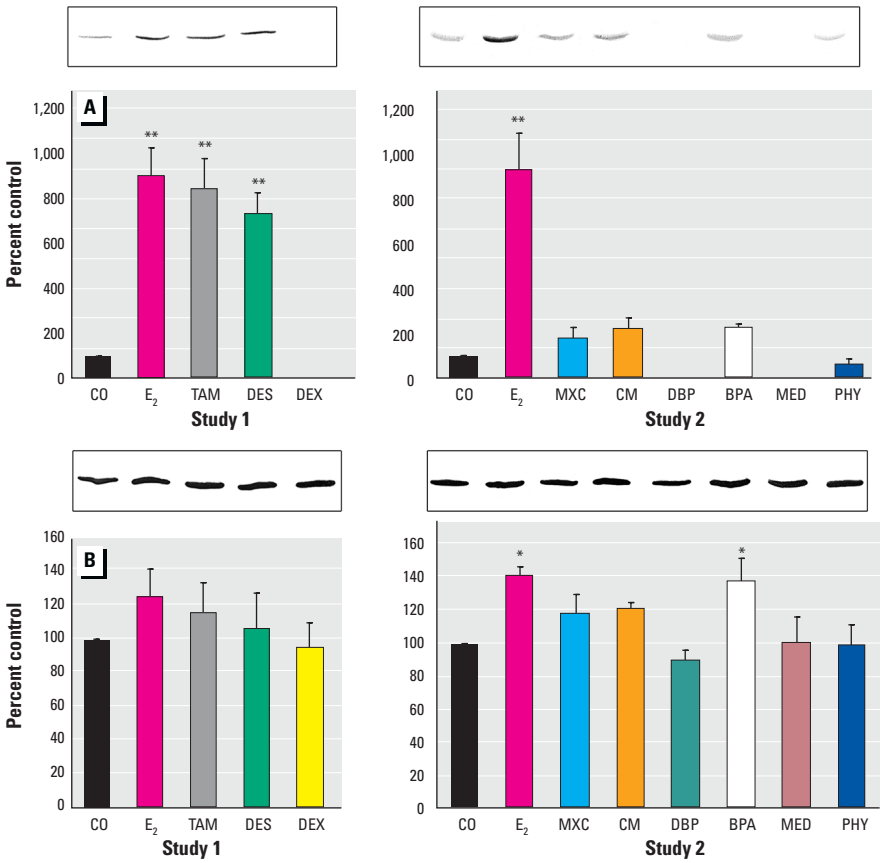

Figure 4. Western analysis of (*A*) hsp72 and (*B*) hsp73 protein levels in 15,000 × *g* homogenate supernatants from pooled uterine tissues of ovariectomized B6C3F1 mice. Animals were treated sc for four consecutive days with CO (vehicle control), E_2 (2 μg/kg/day), TAM (1 mg/kg/day), DES (40 μg/kg/day), DEX (1 mg/kg/day), MXC (100 mg/kg/day), CM (10 mg/kg/day), DBP (5,000 mg/kg/day), BPA (100 mg/kg/day), MED (2 mg/kg/day), or PHY (600 mg/kg/day). The bar graphs represent the results of the densitometric analysis of Western blots of hsp72 and hsp73. Averages from two Western blots for each protein were calculated. The two averages obtained from the two homogenates per treatment were used for calculation of the means and standard deviations, and the results are expressed as percent of control.

*Statistically significant difference from control as determined by one-way ANOVA and the Dunnett's post hoc test ($p < 0.05$).

**Statistically significant difference from control as determined by one-way ANOVA and the Dunnett's post hoc test ($p < 0.01$).

Table 1. Summary of effects on all uterine parameters examined.

Treatment	Uterine weight	Luminal epithelial height	Vacuolar degeneration[a]	Stromal leukocyte infiltration[a]	hsp90α	grp94	hsp72	hsp73
E_2	+++	+++	+++	++	+++	++	+++	+
TAM	+++	+++	NE	++	+++	++	+++	NE
DES	+++	+++	NE	+++	+++	++	+++	NE
DEX	NE	NE	NE	NE	ND	NE	ND	NE
MXC	+	+	NE	NE	++	++	NE	NE
CM	++	+	+++	+	++	++	NE	NE
DBP	NE	NE	NE	NE	NE	NE	ND	NE
BPA	++	+	+	+	++	++	NE	+
MED	NE	NE	NE	NE	NE	NE	ND	NE
PHY	NE	NE	NE	NE	NE	NE	NE	NE

Abbreviations: ND, not detected; NE, no effect.

[a]Ranges are based on frequency of observance: +++, seen in more than 75% of sections; ++, seen in more than 50% of sections; +, seen in more than 0% of sections.

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